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14. ABSTRACT Purpose: To determine the functional role of Translocator Protein (TSPO) in prostate cancer progression. Scope: To demonstrate the effect of TSPO ligands in prostate cancer, we utilized cell proliferation assays, apoptosis ELISAs, and a prostate cancer mouse xenograft study. Our findings provide the first evidence of the anti-tumor effects of lorazepam acting on TSPO. To determine the effect of modulating TSPO expression, we performed overexpression and knockdown studies. These studies provided further evidence that lorazepam is acting through TSPO, as overexpression of TSPO conferred increased susceptibility to lorazepam while TSPO knockdown decreased susceptibility. We investigated the role of TSPO multimers in prostate cancer. TSPO multimers can be induced by reactive oxygen species and may be formed through a di-tyrosine covalent bond. TSPO expression increases with prostate cancer progression. The benzodiazepine lorazepam exerts its anti-cancer effects through its binding to TSPO. Major findings: Collectively, these data suggest that TSPO is an excellent therapeutic target for advanced disease and that our preclinical results demonstrating that the already existing FDA-approved drug lorazepam has anti-tumor effects could be easily translated to the prostate cancer patient population. These studies could lead to a significant change in the management of prostate cancer by providing a treatment option with minimal toxicity for use in advanced disease and could ultimately prevent prostate cancer deaths.					
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Introduction

Translocator Protein (TSPO), previously known as the peripheral benzodiazepine receptor, is a transmembrane molecule that is best known for transporting cholesterol across the mitochondrial membrane for cell signaling and steroid biosynthesis [1, 2]. TSPO has been shown to be overexpressed in numerous malignancies, including those of the breast, prostate, colon, ovary, and endometrium [3-7]. Furthermore, a correlation has been shown between TSPO overexpression and the progression of breast, colorectal, and prostate cancers [8]. Functionally, TSPO has been shown to take part in the regulation of apoptosis through its interactions with the mitochondrial permeability transition pore [9, 10]. TSPO also plays a role in cell proliferation, as a correlation between TSPO expression and cancer cell proliferation has been observed in human astrocytomas [11] and breast cancer [12] while TSPO antagonism inhibits cell proliferation [13-16]. As its former name suggests, the peripheral-type benzodiazepine receptor, now called TSPO, has the ability to bind benzodiazepines with relatively high affinity [17]. Benzodiazepine receptors are found in both the central and peripheral nervous system, but unlike its central-type counterpart, TSPO has no anxiolytic or anticonvulsant effects and has distinct mechanistic and pharmacologic properties [18, 19]. Binding studies have shown that lorazepam and PK11195, the benzodiazepines used in this study, can inhibit binding of the high affinity TSPO ligand Ro5-4864 [20]. Although lorazepam is classically considered a ligand of the central-benzodiazepine receptor, the study by Park et al suggests that it can also bind TSPO. The effects of lorazepam in peripheral tissue, such as the prostate, have yet to be explored.

Body

TSPO expression is increased in human prostate cancer.

To determine relative expression levels of TSPO in human tissue, we performed immunohistochemical analysis of prostate cancer tissue microarrays [21]. As shown in Figure 4A & B-G, we observe significantly increased expression of TSPO in prostatic intraepithelial neoplasia (average score: 3.0/6), primary prostate cancer (4.1/6), and prostate cancer metastases (4.8/6) compared to normal donor (2.0/6), normal tissue adjacent to tumor (2.1/6), and benign prostatic hyperplasia (1.8/6). Furthermore, TSPO expression increases with progression, as prostate cancer metastases have the highest expression levels. Testes and adrenals are steroidogenic tissues documented as having relatively high TSPO expression and were therefore used as positive controls. Increased expression of TSPO is also observed *in vitro* with elevated expression in prostate cancer cell lines PPC-1, DU145, LAPC4, LA98, LNCaP, and LN05 compared to the human embryonic kidney cell line (HEK293), T lymphocytes (Jurkat), a tumorigenic cervical cancer cell line (HeLa), and human hepatocytes (Hep) (Figure 5). It is important to note that the 36kDa band observed is not unique to these studies, as higher molecular weight bands have previously been reported in western blots using antibodies against TSPO [22, 23].

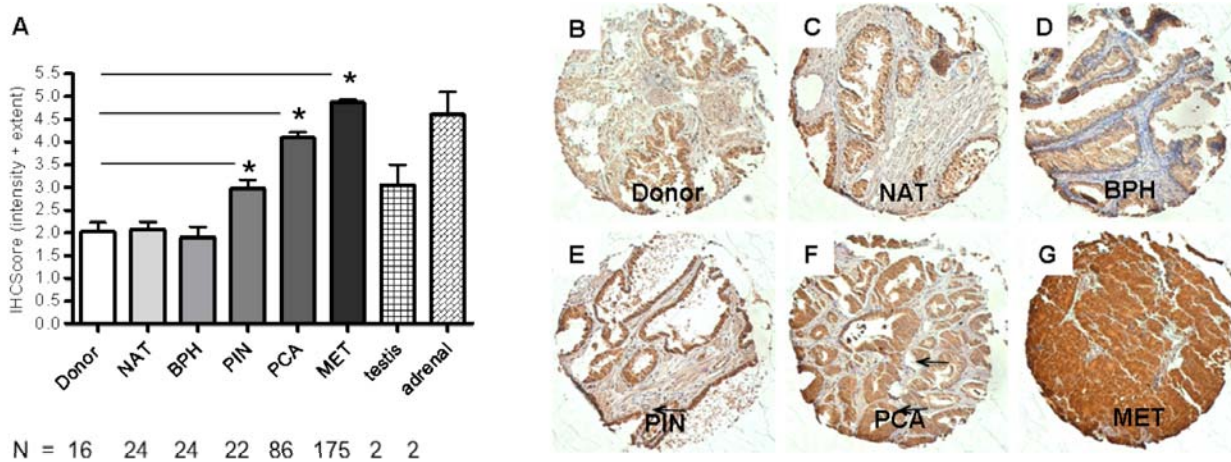


Figure 4. TSPO expression is increased in human prostate cancer tissues.

(A) relative expression of TSPO by immunohistochemistry (IHC) in normal donor prostate (donor), normal prostate tissue adjacent to tumor (NAT), benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), primary prostate cancer (PCA), and prostate cancer metastases (MET) by scoring of TMA cores. (B-G) Representative results of TSPO staining in normal donor prostate (B) NAT (C), BPH (D), PIN (E), PCa (F), and PCa metastasis (G). Arrow indicates PIN and primary prostate cancer glands. * indicates statistical significance $p < 0.05$

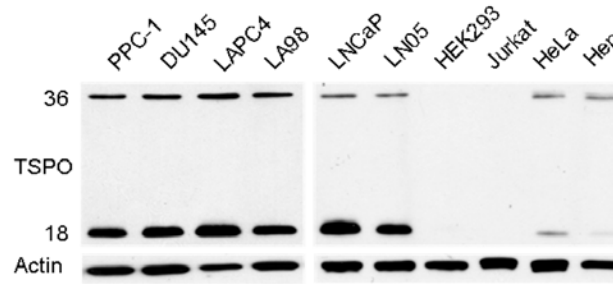


Figure 5. TSPO expression is increased in human prostate cancer cell lines.

TSPO expression in human prostate cancer cell lines PPC-1, DU145, LAPC4, LA98, and LNCaP, LN05 compared to human embryonic kidney cells (HEK293), human T lymphocytes (Jurkat), human cervical cancer cells (HeLa), and human liver hepatocytes (Hep).

Gleason grading is a way to score prostate cancer tissue based on the architecture of the cancerous prostate glands. The Gleason grading scale ranges from very well differentiated cells (grade 1) to very poorly differentiated (grade 5). Two different Gleason grades are assigned to the tissue, representing the primary tissue structure and the secondary tissue structure, for a Gleason score ranging from a possible 2 (1+1) up to 10 (5+5). Analyses of prostate tumor Gleason sum and stage were carried out to identify whether TSPO is altered with disease progression. TSPO levels were high in all tumor specimens compared to normal adjacent glands and TSPO expression increased with increasing grade and stage in the TMA specimens (Table 1). TSPO levels in adenocarcinoma were significantly higher than PIN or NAT when matched for stage except in stage II specimens in which PIN regions demonstrated TSPO levels equivalent to regions of NAT. There was also a significant change in TSPO levels with Gleason sum. High TSPO levels are evident in Gleason ≤ 6 (4.02 ± 0.93) samples compared to the adjacent normal glands (NAT; 2.44 ± 0.63) and increased with Gleason sums 7 (4.16 ± 0.95), Gleason 8 (4.48 ± 0.90) and Gleason 9 (4.58 ± 1.11).

PSA failure is defined as a rise of PSA in the serum following treatment with surgery or radiation. Assessment of TSPO expression in the PSA failure array shows a significant difference in the NAT glands of patients with PSA failure compared with the NAT of patients who remain disease free (Table 1). However there was no difference in the PIN or adenocarcinoma expression of TSPO in the primary tumors of patients with PSA failure compared to disease free patients. The PSA failure array did not contain specimens from patients that have remained disease free, so the samples on the progression array were used for this comparison and matching control tissue was used as comparison between TMAs to assure IHC scoring remained the same across separate arrays.

Table 1. TSPO immunostaining in human prostate tissue by Gleason, stage and PSA failure.

Gleason		<6	7	8	9
		4.02 ±0.93 (n=39)	4.16 ±0.95 (n=81)	4.48 ±0.90 (n=42)	4.58 ±1.11* (n=25)
Stage		PCa	PIN	NAT	
	II	3.97 ±0.86 (n= 19)	3.01 ±1.06 (n = 15)	2.85 ±0.92 (n= 18)	
	III	4.14 ±1.04 (n= 59)	3.52 ±0.95 (n = 19)	2.58 ±0.93 (n = 32)	
	IV	4.46 ±0.88* (n = 23)	3.63 ±0.51 (n = 3)	2.65 ±0.79 (n = 19)	
PSA failure		4.17 ±0.97 (n=50)	3.34 ±0.98 (n=19)	2.66 ±0.88* (n=35)	
disease free		4.02 ±1.13 (n=78)	2.91 ±0.75 (n=17)	2.07±0.82 (n = 23)	

* indicates statistical significance <0.05. Gleason 9 specimens showed higher TSPO levels than Gleason sum 6 and 7 but not Gleason 8. Stage IV PCa had higher TSPO than both stage II and III tumors.

TSPO antagonism has anti-proliferative and pro-apoptotic effects *in vitro*.

We began our preliminary functional studies to identify cancer cell sensitivity to TSPO receptor blockade by screening a series of potential TSPO antagonists, including benzodiazepines temazepam, lorazepam, estazolam, and Ro5-4864, and the isoquinoline carboxamide PK11195. To examine the antagonistic effects of these compounds on cell viability, PPC-1 human prostate cancer cells were treated with these drugs at varying concentrations (0.1-100 μ M). Among all of the compounds examined, the benzodiazepine lorazepam and PK11195 demonstrated the most significant antagonistic properties (Figure 6). Additionally, the effect of these TSPO ligands on cell proliferation was examined and a decrease in cell number following treatment with either PK11195 or lorazepam was observed (Figure 7).

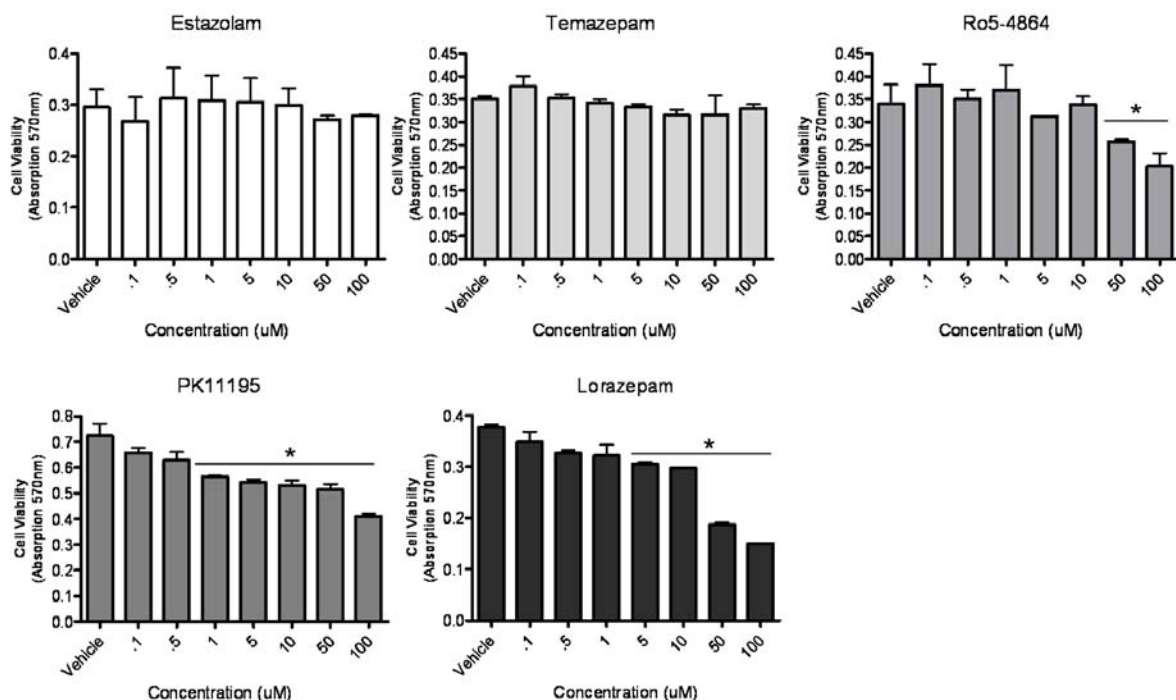


Figure 6. TSPO antagonism decreases cell viability in prostate cancer cells *in vitro*.

MTT assay following 48 hour treatment of PPC-1 with PK11195 or Lorazepam. * indicates statistical significance $p < 0.05$

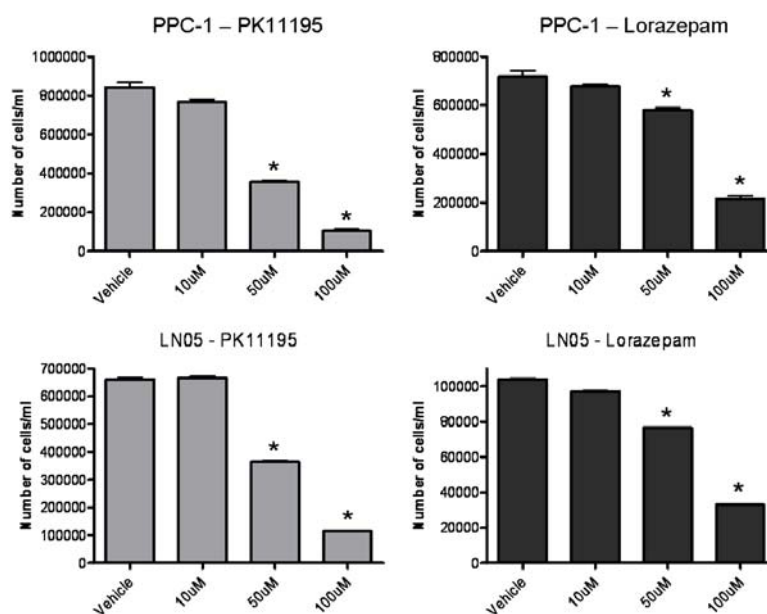


Figure 7. TSPO antagonism decreases cell proliferation in prostate cancer cells *in vitro*.

Direct cell counting of PPC-1 and LN05 cells treated with varying concentrations of PK11195 or Lorazepam or vehicle for 48 hours. * indicates statistical significance $p < 0.05$

In other cancer *in vitro* models, TSPO antagonists have been shown to reduce cell survival through apoptosis. The next experiment was to determine whether the decrease in cell proliferation with treatment with PK11195 or lorazepam was actually due to an induction of apoptosis. PPC-1 cells treated with PK11195 or lorazepam demonstrated a dose-dependent increase in apoptosis following treatment with PK11195 or lorazepam, while LNCaP and LN97 cells only showed significant apoptotic induction at the highest concentration of PK11195 (100 μ M) (Figure 8A). Using Annexin V staining and flow cytometry, a dose-dependent increase in apoptosis in PPC-1 cells treated with PK11195 was also observed (Figure 8B).

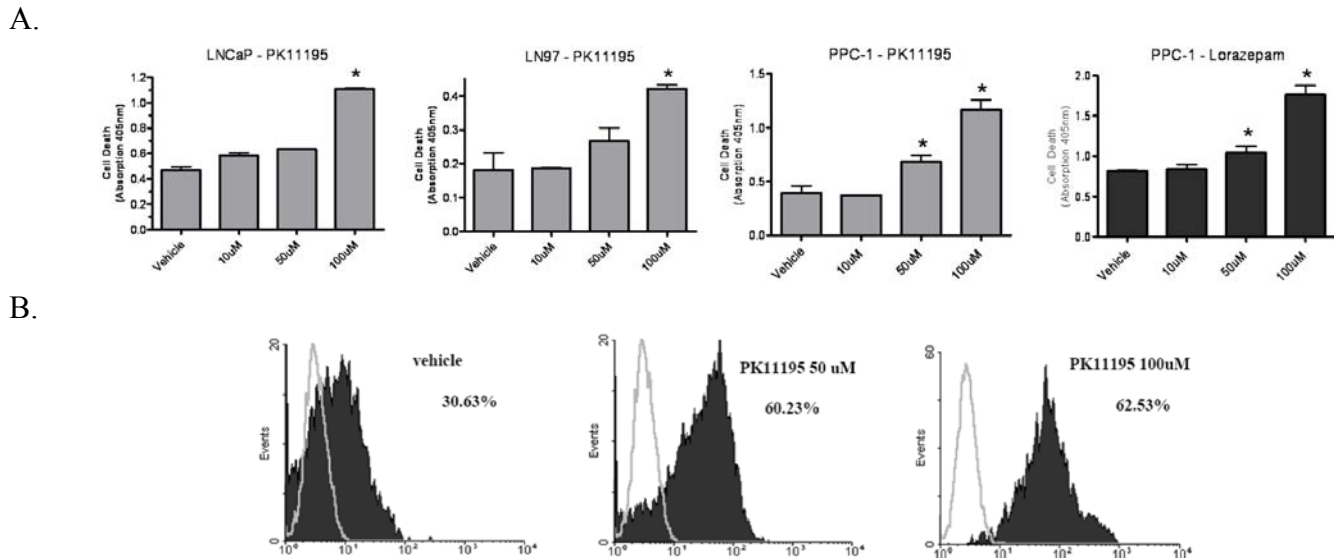


Figure 8. TSPO antagonism increases apoptosis in prostate cancer cells *in vitro*. A. Cell death ELISA following 18 hour treatment with varying concentrations of PK11195 or Lorazepam or vehicle. B. Annexin V based flow cytometry of PPC-1 cells treated with PK11195. * indicates statistical significance $p < 0.05$

TSPO Antagonism Modulates Survival and Cell Cycle Related Proteins

The next goal was to elucidate the cellular signaling pathway by which TSPO ligands were exhibiting their anti-survival and anti-proliferative effects. In a time dependent manner, 50 μ M PK11195 and lorazepam decreased phosphorylation of Akt (pAkt), a protein that is well characterized for its role in cell survival. Lorazepam showed a quicker response, decreasing pAkt levels as early as 15 minutes after treatment (Figure 9). The expression status of cell cycle inhibitor p27 was also investigated. Figure 9 demonstrates an increase in p27 expression 1 hour after treatment with either ligand. Interestingly, p27 levels in PK11195 treated cells went back down to baseline at 4 hours, while p27 levels in lorazepam treated cells remained elevated.

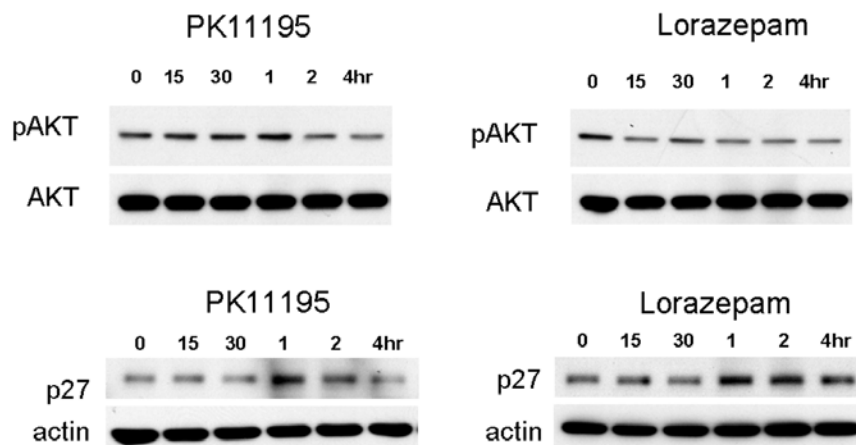


Figure 9. Time course decrease of pAkt and induction of p27 expression by TSPO antagonism.

Immunoblots were reprobed for total Akt (to ensure that treatment with TSPO ligands did not affect total Akt levels) or B-actin (to ensure equal loading). Data are representative of three independent experiments.

***In vitro* analysis of combination TSPO antagonism and Taxotere (docetaxel) treatment in prostate cancer cells.**

Docetaxel is a cytotoxic agent that binds to the B subunit of tubulin, resulting in irreversible polymerization of microtubules. Stabilization of microtubules, which comprise the mitotic spindles, effectively paralyzes the cell in mitosis, leading to the initiation of the apoptotic cascade. Using direct cell counting, the question of whether PK11195 or lorazepam could enhance the cellular response to docetaxel was tested. Studies have suggested that TSPO antagonists may modulate the Akt survival pathway as well as promote opening of the mitochondrial permeability transition pore, the critical step in apoptosis induction. Therefore, we believe that there may be an enhanced effect when TSPO antagonists are combined with Docetaxel. PPC-1 and LN05 cells were treated with 1) vehicle 2) 1nM docetaxel or 3) 1nM docetaxel and PK11195 (or lorazepam) at varying concentrations (1nM-100 μ M) for 48 hours. Figure 10 demonstrates a significant combinatorial effect of docetaxel plus PK11195 but not docetaxel plus lorazepam (compare to Figure 6).

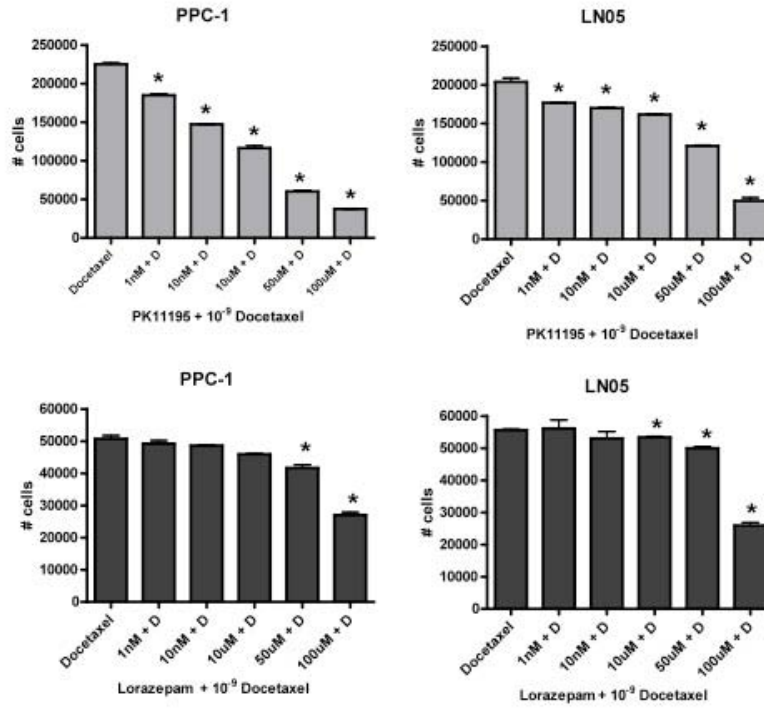


Figure 10. Effect of combination docetaxel + PK11195/lorazepam on prostate cancer cell growth. Direct cell counting of cells treated with docetaxel alone or docetaxel + PK11195/lorazepam D: docetaxel (10⁻⁹) * indicates statistical significance p<0.05

TSPO Antagonism has Anti-Proliferative and Pro-Apoptotic Effects *In Vivo*.

To examine the *in vivo* efficacy of TSPO inhibition, 20 athymic male mice received subcutaneous flank injections of prostate cancer cells. When tumors reached ~100-200 mm³, the mice were randomized into two treatment groups (10 mice per arm) such that each mouse received a daily dose of either 40 mg/kg lorazepam or vehicle (1% DMSO). Tumor measurements were recorded twice a week and mice were euthanized when tumors dimensions reached 2 cm. The tumor measurements demonstrate a divergence in tumor growth between lorazepam and vehicle treated mice: by week nine, lorazepam treated mice exhibited a significantly smaller average tumor volume (2682 ± 539 mm³) when compared to vehicle treated mice (7392 ± 346 mm³) (Figure 11).

A nonlinear mixed effects approach to examine tumor growth longitudinally was implemented to describe the growth characteristics of the PPC-1 cells under vehicle and lorazepam treated arms. The gompertz model resulted in the lowest AIC and objective function values by approximately 25 points under the FOCE Interaction estimation method (p<0.001 for the objective function with 2df). Individually, treatment groups were distinguishable with a covariate representing lorazepam treatment for the kappa (growth rate), gamma (time of maximum growth) and alpha (maximum tumor size) terms (p<0.001) individually. However, once

multiple factors were added, the effect of treatment on the alpha term (i.e., the projected maximum size asymptote for the tumor) was greatest, and the effect on the other two terms were no longer significant. Specifically, the objective function changed from 1791.3 to 1728.8 with the addition of lorazepam treatment as a covariate on the alpha term. This represents a statistically significant change with $p < 0.0001$ for 1 degree of freedom. In addition, the presence of lorazepam resulted in a predicted maximum tumor size approximately $\frac{1}{2}$ as large as that predicted in the presence of vehicle (14900 vs $28400\mu\text{m}^3$).

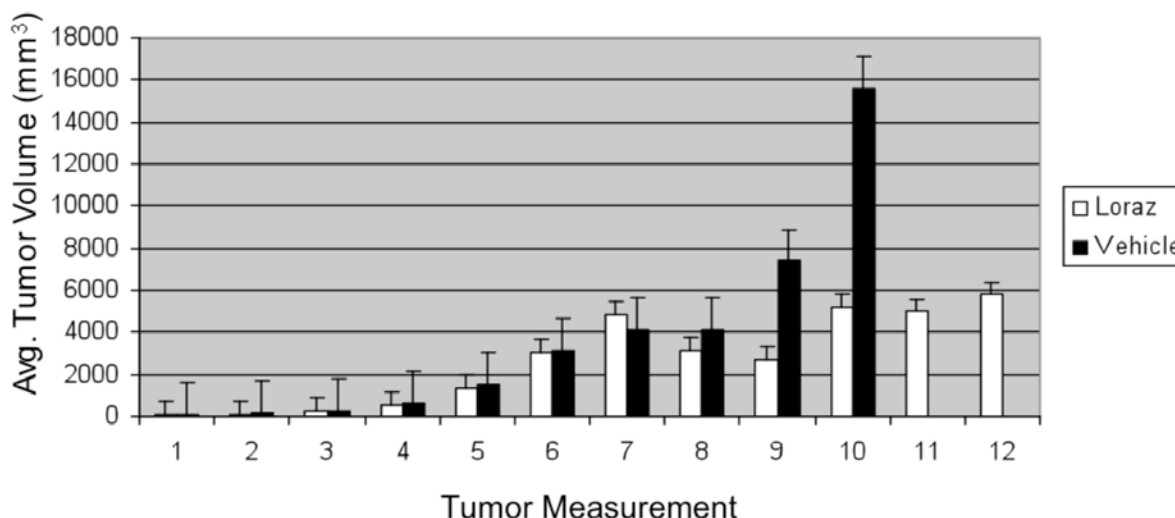


Figure 11. TSPO antagonism decreases average prostate cancer tumor volume over time.

Average tumor volume over time of athymic nude mice bearing PPC-1 xenograft tumors treated daily with Lorazepam (40mg/kg) or 1% DMSO. Tumor volume was measured twice weekly as described in the Materials and Methods section.

Once the tumor burden reached 2 cm, the mice were sacrificed 2 hours after the last dose of vehicle or lorazepam, and tumors were removed and processed for analysis. Tissue sections were stained for TSPO to determine if lorazepam treatment altered TSPO density [24, 25]. Lorazepam had an effect on cell proliferation, as there was a significant decrease in expression of the proliferation-associated protein Ki67 in mice treated with lorazepam compared to the vehicle group (Figure 12). Furthermore, lorazepam treatment did not affect vascularization, as the number of vessels per field was not significantly different between the two groups (Figure 12). TUNEL analysis reveals that lorazepam has pro-apoptotic actions *in vivo*, with the lorazepam treated group having significantly more apoptotic cells compared to the vehicle group (Figure 12).

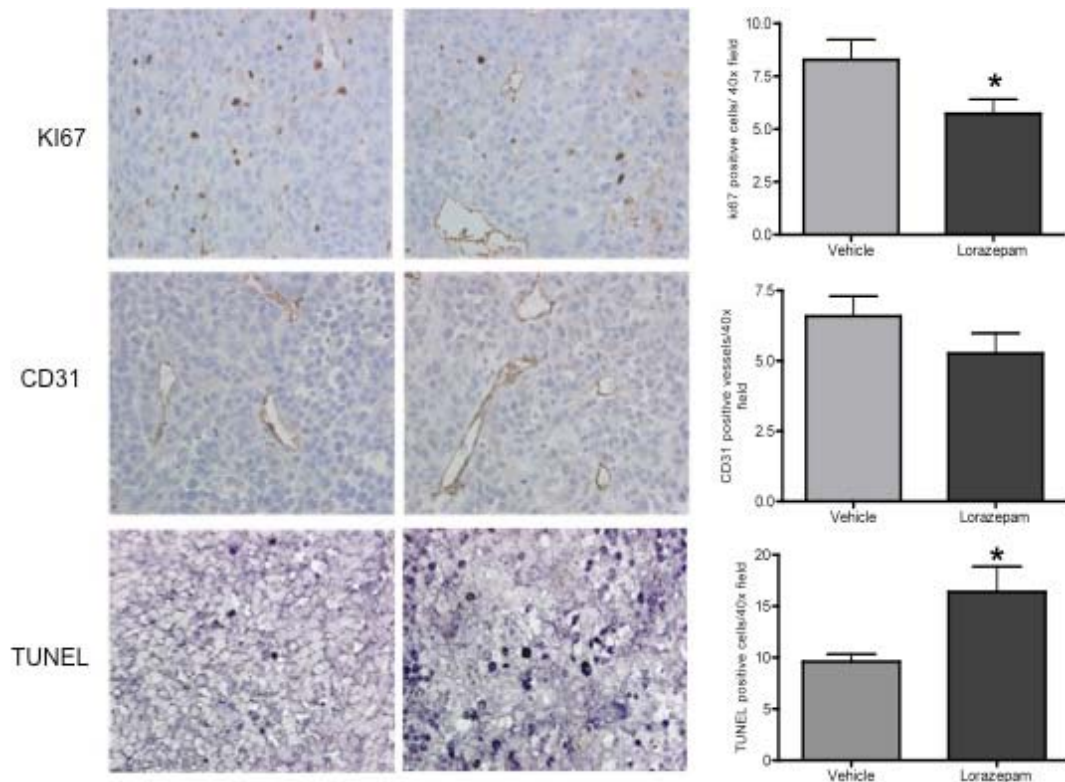


Figure 12. TSPO antagonism decreases cell proliferation and increases apoptosis in prostate cancer cells *in vivo*.

Immunohistochemical staining of lorazepam or vehicle treated PPC-1 xenograft tumors for cell proliferation (Ki67), microvascular density (CD31), and apoptosis (TUNEL). Bars graphs represent average values of positive signal counted in four random fields (40X magnification) * indicates statistical significance $p < 0.05$

Key Research Accomplishments

- Learned numerous techniques both *in vitro* and *in vivo*
- Learned how to ask a scientific question then develop experiments to answer it
- Enhanced my quality of scientific writing through development of this DoD award

Reportable Outcomes

- Some of these data were published in Clinical Cancer Research (October 2009 15; 6177)
- I presented this work at both national (AACR 2009 Denver, CO) and local (University of Pittsburgh 2009 Pittsburgh, PA) scientific meetings.
- In August, I completed my graduate work and received my PhD from the Cellular and Molecular Pathology Program at the University of Pittsburgh

Conclusions

In this series of experiments it was shown that TSPO expression is increased in human prostate cancer. Furthermore, TSPO expression appears to increase with disease progression, as the prostate cancer metastases have the highest expression levels. TSPO is also highly expressed in prostate cancer cell lines, regardless of their androgen-sensitivity. These data support previous reports that TSPO expression is elevated in numerous cancer models, including prostate.

The *in vitro* TSPO antagonism studies presented here reveal that TSPO functions similarly in prostate cancer as it does in previously reported cancer models. Through pharmacologic inhibition, it was shown that TSPO can regulate critical cellular processes involved in transformation such as cell proliferation, cell survival, and cell death. Further studies are required to fully elucidate the molecular mechanisms by which these TSPO ligands are exhibiting their antagonistic effects.

The prostate cancer xenograft mouse study using lorazepam showed for the first time the anti-cancer properties of a benzodiazepine *in vivo*. The effect that lorazepam had on tumor growth over time was quite significant, reducing the tumor volume to half of the size of the vehicle treated tumors. Additionally, immunohistochemistry revealed that lorazepam exhibited similar anti-proliferative and pro-apoptotic properties *in vivo* as it did *in vitro*, evidenced by the decrease in the proliferative marker Ki67 and increase in TUNEL staining, indicative of an increase in cell death. Together, these studies provide additional insight into the role of TSPO as a modulator of apoptosis and proliferation, providing further evidence for its role as a potential therapeutic target for prostate cancer.

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